THE MER MINUS PHENOTYPE, PATIENT RESPONSE TO NITROSOUREAS, AND PROTOONCOGENE ACTIVATION IN HUMAN GLIOBLASTOMAS

Rufus S. Day, III[1], Junji Miyakoshi[1], Kelly Dobler[1], Joan Allalunis-Turner[2], John D.S. McKean[3], Kenneth Petruk[3], Peter B.R. Allen[3], Keith N. Aronyk[3], Bryce Weir[3], Debbie Huyser-Wierenga[4], Dorcas Fulton[4], and Raul C. Urtasun[4]

[1]Molecular Genetics and Carcinogenesis Laboratory, Department of Medicine; [2]Radiobiology Laboratory, Department of Radiation Oncology, and [4]Department of Radiation Oncology, Cross Cancer Institute, 11560 University Avenue, Edmonton, Alberta T6G 1Z2, Canada

[3]Division of Neurosurgery, Faculty of Medicine, University of Alberta, Edmonton, Alberta T6G 2G3, Canada

INTRODUCTION

It is about 10 years since the identification of the first Mer$^-$ line, A172 (Day and Ziolkowski, 1979), a human astrocytoma line produced by Giard, et al.(1973). To review, Mer$^-$ lines are human cell lines defined by their relative inability to support the growth of adenovirus 5 that has been treated with N-methyl-N -nitro-N-nitrosoguanidine (MNNG) prior to infection of cell monolayers (Day, et al., 1980a,b). Such lines lack, without exception, the ability to repair m6Gua produced in their DNA by certain methylating agents (Day, et al., 1980a; Day, et al., 1984), and are thus Mex$^-$ by the definition of Sklar and Strauss (1981). Mer$^-$ strains produced from human tumors are highly sensitive with respect to Mer$^+$ cells as assessed by several endpoints to agents that react with the O^6 of guanine: sister chromatid exchange (Day, et al., 1980a), mutation induction (Baker, et al., 1979, 1980, Domoradski, et al., 1984), cell killing by MNNG or nitrosoureas (Day, et al., 1980a,b; Erickson, 1980a,b; Scudiero, et al., 1984 a,b; Gibson, et al., 1986). The results obtained in cell culture are clear-cut; for example, with MNNG as the damaging agent, the inactivation slopes of survival curves of Mer$^-$ cells are up to 50 fold steeper than are those of Mer$^+$ cells (Scudiero, et al., 1984a). Several groups have inserted parts or all of the E. coli ada gene into Mex$^-$ cells and have provided evidence that such differential sensitivity is likely due to differential repair of m6Gua (Brennand and Margison,

DNA Repair Mechanisms and Their Biological Implications in Mammalian Cells
Edited by M.W. Lambert and J. Laval
Plenum Press, New York

1986, Ishizaki, et al., 1986; Kataoka, et al., 1986; Samson, et al., 1986; Fox, et al., 1987), a point which has been discussed previously, and for which there is substantial evidence (Day, et al., 1987). No matter how persuading the cell culture evidence, there is little evidence to demonstrate that Mer$^-$ cells occur in tumors; i.e., that some fraction of human tumors is composed of Mer$^-$ cells.

We are attempting to demonstrate: 1) whether tumors are composed of Mer$^-$ cells, and 2) an involvement of activation of proto-oncogenes or inactivation of tumor repressor genes in converting cells from Mer$^+$ to Mer$^-$. This paper reviews our work in these areas.

HUMAN MALIGNANT GLIOMAS AS A SUBJECT FOR RESEARCH

Human Brain Tumors. The term malignant glioma refers to astrocytomas with anaplastic foci (AAF; were termed astrocytoma, grade III) and glioblastomas (were termed glioblastoma multiforme or astrocytoma grade IV), but not to astrocytoma grades I or II or to oligodendrogliomas. We are approaching human malignant gliomas in many aspects of our research because:

1. They are a good source of Mer$^-$ cell lines. To date 12 of 37 lines produced from malignant gliomas are Mer$^-$ (Day and Ziolkowski, 1979; Day, et al., 1979; Day, et al., 1980a,b; Scudiero, et al., 1984a; Sariban, et al., 1987; Day, unpublished results). Transformed cell lines can be produced from 20-30% of the glioblastoma biopsies procured (Ponten, 1975; and this paper). Ponten (1975) pointed out that astrocytoma grades I and II, like normal brain tissue, never give rise to permanent lines. Only malignant gliomas give rise to permanent lines. Therefore, a permanent line must have arisen from tumor tissue. Of course, tumor tissue that does not produce a permanent line is not necessarily normal. The work of James, et al.(1988), who found loss of heterozygosity for loci on chromosome 10 in 28 of 29 glioblastoma biopsies, shows that their biopsy material is largely tumor material.

2. Brain tumors are difficult to treat successfully (see, for example Wilson, 1976), so that research inroads may more likely be clinically useful than for other tumors.

3. Brain tumors are one of the few human tumors which are treated with single agent chemotherapy rather than the multi-agent regimens proscribed for many other tumors. The single agent is usually a nitrosourea, to which Mer$^-$ cells are sensitive in vitro. Initial post-diagnostic treatment at the CCI entails surgery followed by radiation therapy. CCNU is given upon relapse, and thus treatment with CCNU is isolated in time from other treatments, and the response of the tumor to CCNU may be followed by CT scan with few complications.

4. There is a growing literature on the genomic changes in human glioblastomas as detected at 1) the cytological level in short and long term cell cultures of astrocytoma/ glioblastoma biopsies (see for example, Bigner, et al., 1988); the level of proto-oncogene activation as observed in biopsies and longer term cultures (Kinzler, et al., 1987; Wong, et al.,

1987); and the level of loss of heterozygosity (James, et al., 1988).

Specific questions that we are approaching are:

1. Does patient response (or resistance) to CCNU [3-cyclohexyl-1-(2- chloroethyl)-1-nitrosourea, a nitrosourea to which Mer$^-$ lines are sensitive, Erickson, et al., 1980a] correlate with the Mer phenotype of the cell line developed from the surgically removed biopsy? Do short term (3 - 5 day) tests, in which cells from disaggregated biopsies are treated with either radiation or BCNU and are assayed for survival, reflect the behavior of the line produced?

2. Do plasmids containing activated oncogenes convert Mer$^+$ cells to Mer$^-$?

3. Are there patterns of activation of proto-oncogenes or inactivation of tumor suppressor genes that suggest their involvement in producing the Mer$^-$ phenotype?

4. Are there nucleic acid sequences from Mer$^-$ brain tumor cells that are not contained in Mer$^+$ cells or vice versa?

PATIENT RESPONSE AND THE MER MINUS PHENOTYPE

In the two years of this collaborative effort, 37 biopsies have been obtained from brain tumor patients seen both at the Cross Cancer Institute/University of Alberta Hospital system and at the Royal Alexandra Hospital, both in Edmonton (Table 1). These were cultured by three methods:

1) Biopsies were washed with PBS, cut with crossed scalpels into fragments of volume less than 1 mm^3, which were placed in 60 mm culture dishes. To ensure attachment to the dish, coverslips with stopcock grease (on diagonally opposing corners) were pushed gently on top of the fragments. The cultures were supplemented with either F12, Dulbecco's Modified Eagle Medium (DMEM), or Biorich medium [all with 10% fetal calf serum (FCS)].

2) Washed biopsies were shaken in a 50 ml tube with 20 ml 0.5% trypsin plus 33 units/ml collagenase in PBS at 37 degrees. The small clumps and single cells were harvested twice, after 20 minutes and 40 minutes of treatment, washed 2x with PBS and plated in 60 mm dishes with medium as above.

3) This is the method of Freeman and Hoffman (1986). Spongostan was cut into 1 cm x 1 cm x 0.5 cm pieces, one which was placed in each 35 mm dish with one of its 1 cm x 1 cm sides contacting the plate surface. Medium (as above) was added such that 1 mm of the Spongostan was not submerged (but medium was maintained in this portion by capillary action), and 1 to 4 biopsy fragments were placed on the protruding Spongostan. In all methods, plates were kept at 35-37 degrees at 85% humidity, and received medium changes weekly. We have prepared 8 transformed lines from 30 biopsies, 7 by method 1 and 1 by method 2 which was introduced at biopsy 16.

Table 1. Patients of the brain tumor study.

#	Age Sex	Diagnosis	Mer*	Response to Therapy (through 1 Aug 88)
1	13F	Oligodendroglioma		No CCNU (RT only; no recurrence)
2	58M	Glioblastoma	+	No CCNU (died during treatment with RT)
3	55M	Oligodendroglioma		No CCNU (RT only; no recurrence)
4	51M	AAF		2 courses CCNU; adverse change on CT after 1st course.
5	30F	Grade I-II Astrocytoma		No CCNU (RT only; no recurrence)
6	67M	Glioblastoma	-	No RT; 4 courses CCNU; resp. to 1st 2 cycles; died 28 Feb 1988.
7	47M	Glioblastoma	+	CCNU
8	41M	Benign Meningioma		No CCNU
9	58F	Meningioma		No CCNU
10	49M	Glioblastoma	-	No CCNU (RT only; no recurrence)
11	66M	Glioblastoma	+	No CCNU
12	53M	Grade III Astrocytoma		No CCNU (recurrence in March 88; pt. refused CCNU)
13	21M	Recurrent Astrocytoma		4 Cycles CCNU; enhancing area unchanged or improved on CT; died July 88.
14	36M	Glioblastoma		CCNU - 1 course; no response; repeat resection and RT in Fall 87
15	41M	AAF		No CCNU.
16	63M	Glioblastoma	+	No CCNU (RT only; pt. moved to Oregon April 88; no recurrence)
17	68M	Glioblastoma		No CCNU (died during RT)
18	54M	Glioblastoma		RT + IUdR (no recurrence)
19	17M	Ganglioglioma		No CCNU
20	46M	Grade II Astrocytoma		No CCNU (RT only; no recurrence)
21	40M	Glioblastoma		RT + IUdR (no recurrence)
22	24M	Germ Cell Tumor		No CCNU (RT only; no recurrence)
23	58M	Glioblastoma		CCNU (1st course given July 88)
24	29M	AAF		No CCNU (RT only; no recurrence)
25	51M	Metastatic Adenoma		No CCNU
26	35M	Grade II Astrocytoma		No CCNU (RT only; no recurrence)
27	67M	Glioblastoma	-	No CCNU (? recurrence; may start CCNU)
28	70M	Glioblastoma		No CCNU (refused treatment of any kind)
29	51M	Glioblastoma		No CCNU (RT only; no recurrence)
30	35M	AAF		No CCNU (RT + IUdR only; no recurrence)
31	61F	Glioblastoma		No CCNU (no treatment with RT or CCNU poor prognostic factors + pt. choice)
32	47M	Glioblastoma		No CCNU (finished RT 1 month ago)
33	37M	Grade II Astrocytoma		No CCNU (currently on RT)
34	36F	Glioblastoma		No CCNU (currently on RT)
35	25M	Grade II - AAF		No CCNU (currently on RT)
36	30M	Astro/glial hyperp.		No CCNU
37	17M	Grade II Astrocytoma		No CCNU (currently on RT)

*Mer phenotypes reported for transformed cell lines only

Note: tumors other than malignant gliomas were included during preliminary work.

Using method 1, two patterns of outgrowth of a transformed line were observed. Biopsies from patients 2, 6, 16, and 27 grew directly into transformed lines on all plates (12 to 16 plates prepared per biopsy) with little or no apparent growth of non-transformed cells. Biopsies from patients 7, 10, 11, and 12 grew into strains on 1 or 2 of the 12 to 16 original plates, and appeared after the outgrowth of non-transformed cells. No other biopsy has yet produced a transformed line. Method 2, introduced on receipt of the biopsy from patient 16, was successful in producing a transformed line from patient 16, but from no other biopsy. Method 3 was used for biopsies 1-4, and is being reintroduced as a replacement for method 2. Biopsy material from several grade IV astrocytoma (glioblastoma) patients has been in culture for more than 1 year, and has not yet produced transformed lines. Strains produced from patients 2, 6, etc., were given the names M002, M006, etc.

Of the eight transformed lines, seven are from grade IV astrocytomas (4 Mer^+: M007, M011, and M016; 3 Mer^-: M006, M010, and M027), and one from a grade III astrocytoma (Mer^+).

As yet limited patient response data is available. Six patients have received CCNU: patients 4, 6, 7, 13, 14, and 23. No response data is available for patients 7 or 23. Patient 14 was resistant as judged by CT scan. Patients 4, 6, and 13 were sensitive, showing tumor regression or no progression. Patient 6 was unique in that radiation treatment was refused; the patient received oral CCNU only. In patient 6 the response to CCNU correlated positively with the Mer^- phenotype of the cell culture derived from the tumor.

We have compared the MTT assay (Mosmann, 1983) as a short term test of cytotoxicity with the clonogenic assay used previously (Scudiero, et al., 1984b). Our data indicate that cellular sensitivity to BCNU of a disaggregated biopsy, as assessed by the MTT assay, is a good indicator of BCNU sensitivity of the line produced from that biopsy, as assessed by a clonogenic assay.

PLASMIDS WITH ACTIVATED PROTOONCOGENES HAVE NOT YET PRODUCED THE MER^- PHENOTYPE

We have prepared three plasmids bearing activated oncogenes.

1) The src gene was selected because it appeared that Rous sarcoma virus converted a human osteosarcoma cell line, HOS, a clone of TE85, to a Mer^- strain RHOS (Yarosh, et al., 1984b). The following experimentation shows why we now suspect that this finding is erroneous. We obtained the plasmid pMS484c (Jakobovits, et al., 1984) as a kind gift from H. Varmus, and cloned its 2.8 kb Bam HI fragment containing both v-src-RSV and the viral 3' LTR into pRSV deltalinker Neo (our construct) to obtain pRSVsrcNeoI (Figure 1). This construct contains both an RSV-LTR driven v-src gene and an RSV-LTR driven neo gene. pRSVsrcNeo was transfected into HOS cells by polybrene mediated transfection (Aubin, et al.,1988), and colonies stable to selection by 200 ug/ml G418 in F12 medium

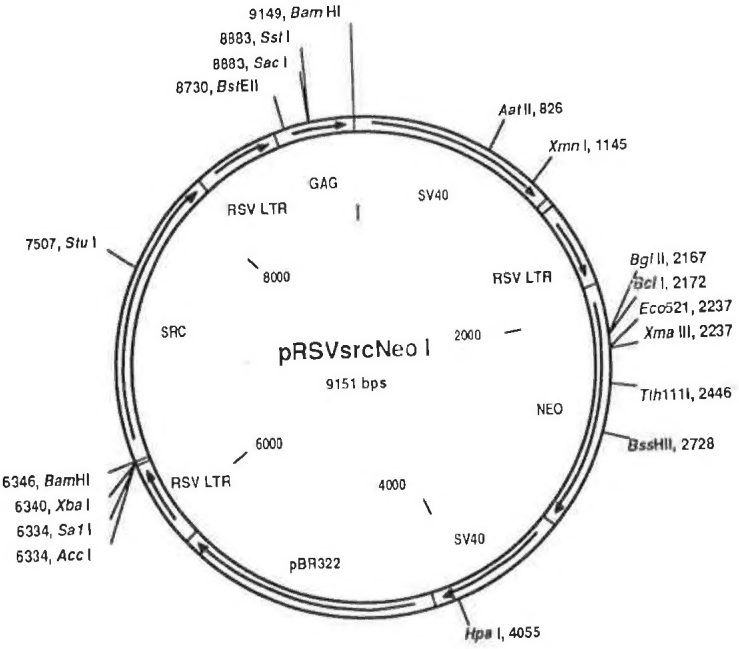

Figure 1. Plasmid pRSVsrcNeo I

were selected, cloned, grown into cell strains, and tested for their Mer phenotype. In summary, 4 of 78 clones prepared in this manner were Mer$^-$. Mer$^-$ clones were found only in uncloned HOS cells; no Mer$^-$ clones were observed when HOS clone 8 (selected as a clone with flat cells) was used as a host for transfections. Furthermore, when selected transfected clones were grown in the absence of G418 and tested for expression of their src gene by Northern and dot blotting, no correlation between expression of the src gene and Mer phenotype was observed. In Figure 2, HOS CW3 and HOS AC12 are Mer$^-$; the remaining strains are Mer$^+$. Only HOS D, HOS CA, HOS CS, and HOS CV show expression of the src gene, detected by a src gene probe. Control blots using the gamma actin gene as a probe (Figure 3) show roughly equal expression of this gene. In a similar protocol, we observed no Mer$^-$ transformants due to transfection of HOS-8 by c-hu-myc gene (exons II and III) placed downstream from an RSV promoter. We did not analyse the strains by blots.

2) We examined the expression of the SV40 genes in Mer$^+$ and Mer$^-$ cells that were produced by SV40 transformation of Mer$^+$ human fibroblasts. The supposition was that cells expressing greater levels of a SV40 mRNA or any amount of a given mRNA species would more likely be Mer$^-$ than those cells producing lesser amounts of SV40 mRNA. However, Figure 4 shows that there is no obvious relationship between SV40 mRNA production and Mer phenotype SV40. Strains IMR90-830, IMR90-890, SV80, XP12T703, GM638, WI38VA13, W18VA2, and CRL1584 are Mer$^-$ strains; AT5BIVA2, W98VA1, WI26VA4, and GM637 are Mer$^+$.

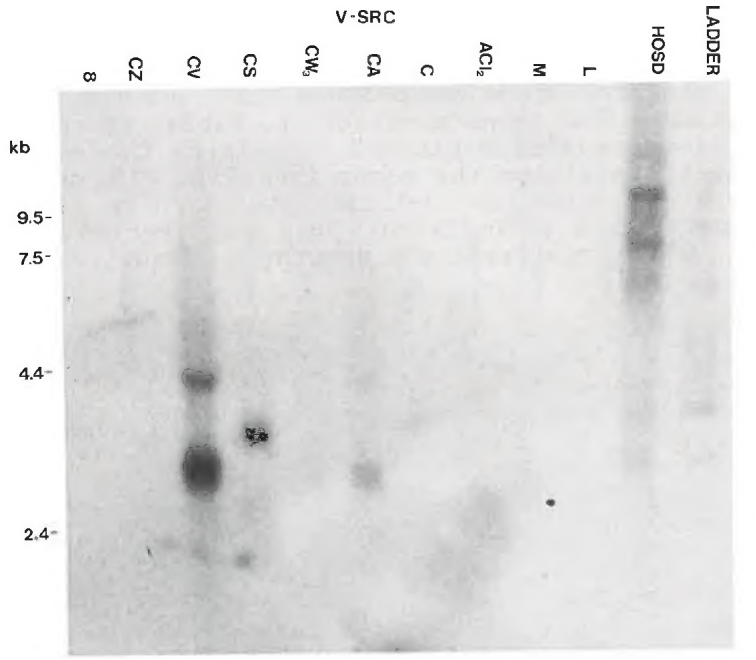

Figure 2. src Specific mRNA Production by pRSVsrcNeo I. Transformed HOS Clones. HOS clones D, L, AC12, M, C, CA, CW3, CS, CV, CZ were selected with G418 and passaged without G418 for 10 to 50 generations prior to RNA extraction. HOS8 is a clone of HOS selected without transfection and in the absence of G418.

A Southern blot, not shown, shows that there is no specific integration site associated with the production of Mer$^-$ cells. We can not conclude that SV40 insertional inactivation of the Mer gene is impossible. Such inactivation could occur because SV40 transformants occur at approximately 0.0003 per cell infected. If, as is common, an input multiplicity of 100 SV40 genomes per cell were used, and if there are a million genes per cell, three "target genes" of which, when inactivated give rise to transformation, and if each infecting SV40 genome were to insert randomly, 0.0003 transformants per cell infected would be generated. According to this idea, two of the three target genes when inactivated would be supposed to generate the Mer$^-$ phenotype (in a dominant fashion) to account for the fact that 2/3 of the transformants are Mer$^-$. On the other hand, SV40 is known to transform human cells by a "two step" mechanism. An SV40 transformed "focus" goes through a growth crisis (at approximately the same doubling as non-infected parent fibroblasts do) from which a permanent cell line may or may not arise. Perhaps it is this second, immortalizing, event that generates the Mer$^-$ phenotype.

3) The adenovirus transformed human embryonic kidney (HEK) cell line 293 is another Mer$^-$ cell line. All primary HEK strains (3) and one HEK line (Flow 4000) that we have tested are Mer$^+$. We presume, but have not proved, that adenovirus transformation can produce Mer$^-$ cultures from Mer$^+$ cultures as does SV40 transformation. L. Babiss (Rockefeller) kindly provided us with a plasmid containing the adenovirus XhoC fragment containing the adenovirus E1A, E1B, and 55 kD terminal protein genes (pMK 0-15.5) into which we inserted a neo gene to produce pMKO-15neoI. This was transfected into A549 cells, known to support the growth of adenoviruses well.

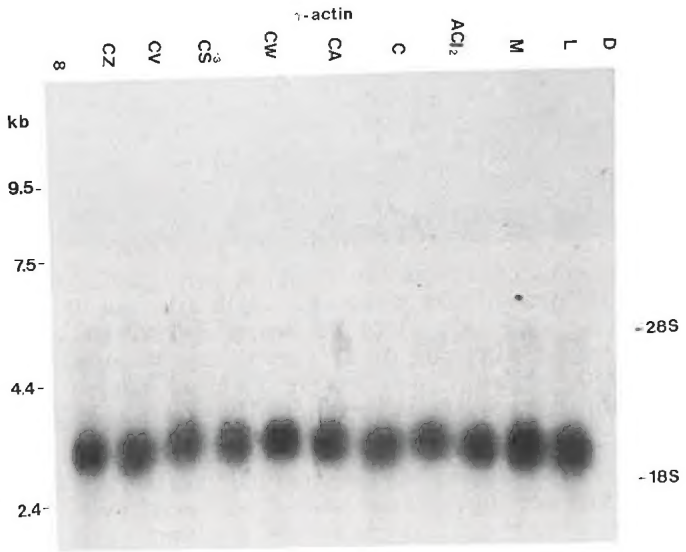

Figure 3. Gamma actin - Specific mRNA Production by the HOS Clones in Figure 2.

Selected clones, maintained in G418 at 350 ug/ml in F12, were tested for their ability to express the E1A product. This was done by assaying the plaque forming activity of the adenovirus 5 deletion mutant Ad5d1312, shown to require the adenovirus early products for growth (Jones and Shenk, 1979) and kindly supplied to us by T. Shenk. Of 10 clones tested, 2 promoted plaquing by Ad5d1312, showing that these are producing functional E1A protein. Initial tests show that these strains are Mer$^+$. We are passaging these cells in increasing G418 concentrations in attempts to enhance E1A expression and possibly produce a Mer$^-$ cell.

Because the Mer$^-$ phenotype accompanies transformation, we believe that either the activation of a gene involved in transformation (or the loss of function of that gene) may pro-

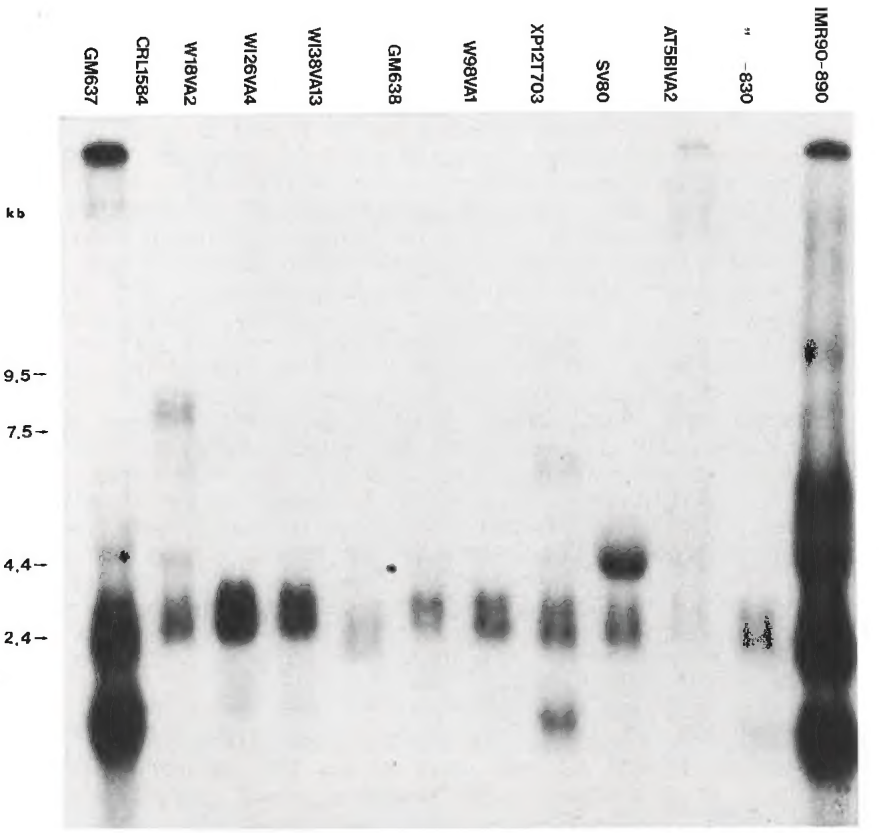

Figure 4. SV40-Specific mRNA production by SV40-Transformed Mer$^+$ and Mer$^-$ Cell Lines. Strains GM637, W126VA4, W98TA1, and AT5BIVA2 are Mer$^+$; CRL1584, W18VA2, WI38VA13, GM638, XP12T703, SV80, IMR90-830, and IMR90-890 are Mer$^-$. Probe was entire SV40 genome from pBRSV. (Southern blots showed a similar pattern).

duce transformed Mer$^-$ cells from nontransformed Mer$^+$ cells. The fact that the Mer$^-$ phenotype is most often dominant in Mer$^+$/Mer$^-$ hybrids (Yarosh, et al., 1984b; Day and Dobler, unpublished; but see Ayres, et al., 1982), argues that the Mer$^+$ phenotype is inactivated by activation of a gene during

transformation, possibly by a transforming gene or oncogene.

We have done a preliminary study of proto-oncogene activation in 5 Mer$^+$ and 5 Mer$^-$ cell lines from human glioblastomas: The Mer$^+$ strains are: SAN, MIL, U118MG, GRE, and T98; the Mer$^-$ strains include A172, CLA, RIC, U87MG, and P4. The origins of all but P4, which was from Drs. Kornblith and Smith (NIH) are published (Day and Ziolkowski, 1979; Day, et al, 1980a,b; Scudiero, et al., 1984 a,b).

EGFR gene. The first proto-oncogene reported to be altered in human glioblastomas was the epidermal growth factor receptor (EGFR) gene (Libermann, et al., 1985). The EGFR gene is a 26 exon, 110 Kb gene specifying 5.1 and 9.5 kb transcripts that encode a 1126 amino acid, 175 kd product. EGFR has a tyrosine specific kinase activity, an ATP binding site, and binds both EGF and transforming growth factor alpha (Haley, et al., 1987). The gene is on chromosome 7, which is often present in three copies in glioblastomas (Bigner, et al., 1988). It was amplified and/or overexpressed in 4 of 10 primary glioblastomas (Libermann, et al., 1985). More recently 24 of 63 gliomas were found to have both amplification and elevated mRNA expression, while none of the remaining glioblastomas showed either sign of EGFR gene activation (Wong, et al., 1987). In cell lines, 2 of 6 cell lines from glioblastomas showed amplification of and short deletions within the EGFR gene (Yamazaki, et al., 1988). In our work, two strains of 17 glioblastomas show an altered EGFR allele by Southern analysis (see Figure 5) using plasmid pE7 (exons 2-21) as the source of the probe. CLA appeared to suffered a deletion, while A172 contained an insertion. Other differences in the 10 kb region may be RFLPs because no consistent differences were observed with EcoRI or PstI cut genomic DNAs as were with CLA and A172. The Hind III 5.3 kb RFLP was ob-

Table 2. Dot Blot analysis of mRNA Production by Mer$^+$ and Mer$^-$ Malignant Glioma Cell Lines for Selected Genes.

Cell line/ Strain	(Mer)	Her-2/ NEU	EGFR	c-Ha-ras	c-myc	v-src	c-raf	c-sis	gli	γ-actin
A172	(-)	++	+++	+++	+	+	++	+/++	+	++
U87	(-)	-	+/++	+++	+/++	+	++	+/++	+	++
RIC	(-)	+	+	+++	+	+	++	+	+	+
P4	(-)	++	+++	+++	+	+	++	+	+	++++
CLA	(-)	+	+++	+++	+	-/+	++	+/++	+	+++
T98	(+)	+	+	+++	+/++	+/++	++	+/++	+	-/+
SAN	(+)	++++	-/+	+++	+	++	++	+/++	+	+
118MG	(+)	+	++	+++	+	+/++	++	+/++	+	+
GRE	(+)	+	++	+++	+	+	++	+/++	+	+++
MIL	(+)	+	++	+++	++	++	++	+/++	+	+++
CCL2	(+)	+	NT	+++	++++	+	++	NT	NT	++
HOS8	(+)	+	NT	+++	-/+	-/+	++	NT	NT	++
KD	(+)	++	+++	+++	-/+	NT	++	+/++	+	++
A431	(+/-)	+++	++++++	NT	NT	NT	NT	NT	+	NT

(pluses are approximately linearly related to mRNA production; NT, not tested)

served: none of 6 analyzable Mer$^-$ glioblastoma strains contained the 5.3 kb band, whereas 6 analyzable Mer$^+$ strains did. One Mer$^+$ glioblastoma line was observed not to produce little or no EGFR mRNA, whereas 10 Mer$^+$ and Mer$^-$ lines did (See Table 2).

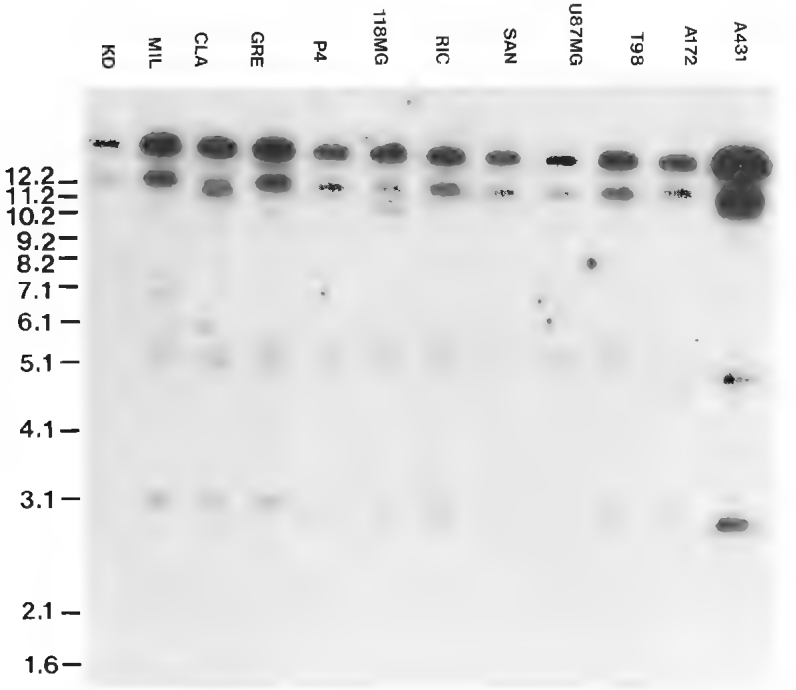

Figure 5. EGFR gene in Human Glioblastoma Cell Lines. Genomic DNAs were digested with HindIII, electrophoresed, blotted, and probed with the EGFR insert in plasmid pE7. A431 is known to have an amplified EGFR gene and KD is a normal fibroblast cell strain.

C-sis gene. Another proto-oncogene expressed in human glioblastomas is the platelet-derived growth factor beta chain (PDGFB) gene (Nister, et al., 1988), whose product is structually similar to the v-sis product of simian sarcoma virus (Waterfield, et al., 1983), a virus that causes gliomas in primates (Deinhardt, 1980). In these studies, all of 21 lines tested expressed PDGF A chain mRNA, 16 of 21 expressed the PDGF B chain mRNA and PDGF receptor mRNA was expressed in 15-16 of the 21 lines. A mechanism involving autocrine stimulation of malignant growth is suspected, but not proved (Nister, et al., 1988). In our studies no extra chromosomal material was seen in dot blot and Southern analysis of EcoRI, HindIII, and PstI cut genomic DNA from 34 transformed and 2 non-transformed strains. The Hind III RFLP pattern was

analysed with the pSM-1 probe: 22 strains had a 30 kb band only, 5 strains had a 14 plus a 6 kb band, and 9 strains contained all these bands. There was no correlation of the RFLP pattern with Mer phenotype.

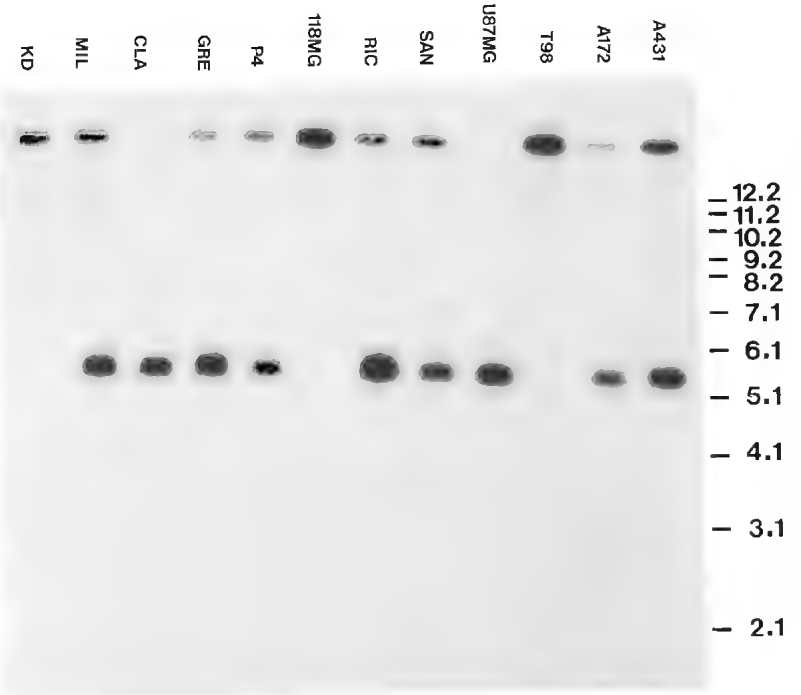

Figure 6. gli gene in Human Glioblastoma Cell Lines. Genomic DNAs were digested with EcoRI, electrophoresed, blotted, and probed with the gli insert in plasmid pKK36P1.

gli gene. The gli gene was isolated from a glioblastoma cell line with double minute chromosomes using a library of sequences isolated by selecting for amplified DNA. The gene is amplified and overexpressed in the cell line, D-259MG, used to isolate it and in 1 of 63 primary glioblastomas (Wong, et al., 1987; Kinzler, et al., 1987). To our knowledge there is no literature published on studies of the gli gene in cell lines. In our work (see Figure 6) an apparently new restriction length polymorphism (RFLP) was detected with a gli gene probe (kindly supplied by B. Vogelstein) by Southern analysis of Eco RI cleaved genomic DNAs isolated from 39 strains. 19 strains had only a 15-16 kb band; 3 had only a 5.3 kb band, while 17 had both. Patterns in 10 tumor strains were consistent with extra chromosome 12 material in the region of gli. There was no correlation between either the

polymorphism or the extra gli material and Mer phenotype.

ROS1 gene. In a study of 45 lines produced from many solid tumor types, the ROS1 gene on chromosome 6 was found to be expressed to moderate or high levels almost solely by astrocytomas and glioblastomas (Birchmeier, et al., 1987). In one glioblastoma, U118MG, the ROS1 gene was altered in a way suggesting activation. It was found to be truncated, likely due to an intrachromosomal deletion event, and gave a product lacking the extracellular receptor site, but retaining tyrosine kinase activity and the transmembrane region (Wigler, personal communication). We have not yet studied the ROS1 gene, although published data on ROS1 gene mRNA production (Birchmeier, et al., 1987) does not appear to correlate with the Mer$^-$ phenotype of the same strains as determined in our laboratory.

Interferon (IFN) genes. A possibly telling hint about Mer$^-$ glioma lines is their sensitivity to growth inhibition by interferons (IFNs) alpha and beta (Cook, et al., 1983; Yarosh, et al., 1985). IFN alpha and beta genes have not yet been shown to be altered in brain tumors. However, the p arm of chromosome 9 in glioblastomas frequently is a breakpoint for translocations and deletions (Bigner, et al., 1988), the location the genes for IFN alpha and beta (Shows, et al., 1982). Furthermore, the c-ets-1 gene on chromosome 6 is translocated to the interferon region in chromosome 9 in human acute monocytic leukemia (Diaz, et al., 1986). The finding that some patients with acute lymphoblastic leukemia or non-Hodgkin lymphoma have a homozygous deletion of the IFN-alpha and -beta genes (Diaz, et al., 1988) and the possibility that IFNs may act as tumor suppressor genes (see, for example, Reznitzky, et al., 1986) supports the hypothesis that deletion of IFN genes in brain tumor cell strains may constitute an oncogenic event. We are currently testing this possibility. We have found that the IFN-beta gene is deleted from several of our human brain tumor lines, using as probe the IFN-beta gene of Mark, et al., (1984).

Protooncogene expression. Northern and RNA dot blotting was used to assess the expression of mRNAs of selected protooncogenes in five Mer$^+$ and five Mer$^-$ strains. The results are shown in Table 2. Although there are significant differences among the strains, there is no correlation of expression of any one gene with Mer phenotype.

CONCLUSIONS

We have begun a patient study in order to assess whether patient tumors behave as if they are composed of Mer$^-$ cells, defective in the repair of O^6-methylguanine. This study is promising in that a tumor from one patient both responded to CCNU chemotherapy and gave rise to a Mer$^-$ line. In addition, to determine possible relationships between gene activation and Mer phenotype, we have begun a study of gene expression and structure in Mer$^+$ and Mer$^-$ lines either produced by SV40 transformation or from human brain tumor biopsies. This study has led to interesting findings about brain tumors, but has not led us to understand the basis for the Mer$^-$ phenotype. A study of the mRNAs differentially expressed by Mer$^+$ and Mer$^-$ strains may lead us further.

REFERENCES

Aubin, R., Weinfeld, M., and Paterson, M.C., 1988, Factors influencing efficiency and reproducibility of polybrene-assisted gene transfer. Somatic Cell and Mol. Genet., 14:155.

Ayres, K., Sklar, R., Larson, K., Lindgren, V., and Strauss, B., 1982, Regulation of the capacity for O^6-methylguanine removal from DNA in human lymphoblastoid cells studied by cell hybridization. Mol. Cell. Biol., 2:904.

Baker, R.M., Van Voorhis, W.C., and Spencer, L.A., 1979, HeLa cell variants that differ in sensitivity to monofunctional alkylating agents, with independence of cytotoxic and mutagenic responses, Proc. Natl. Acad. Sci. USA, 76:5249.

Baker, R.M., Zuerndorfer, G., and Mandel, R., 1980, Enhanced susceptibility of a xeroderma pigmentosum cell line to mutagenesis by MNNG and EMS, Environ. Mutagenesis, 2:269.

Bigner, S.H., Mark, J., Burger, P.C., Mahaley, M.S., Jr., Bullard, D.E., Muhlbaier, L.H., and Bigner, D.D., 1988, Specific chromosomal abnormalities in malignant human gliomas. Cancer Res., 88:405.

Birchmeier, C., Sharma, S., and Wigler, M., 1987, Expression and rearrangement of the ROS1 gene in human glioblastoma cells. Proc. Natl. Acad. Sci. USA, 84:9270.

Brennand, J., and Margison, G.P., 1986, Reduction of the toxicity and mutagenicity of alkylating agents in mammalian cells harboring the Escherichia coli alkyltransferase gene. Proc. Natl. Acad. Sci. USA, 83:6292.

Cook, A.W., Carter, W.A., Nidzgorski, R., and Akhtar, L., 1983, Human brain tumor-derived cell lines: growth rate reduced by human fibroblast interferon. Science, 219:881.

Day, R. S., III and Ziolkowski, C.H.J., 1979, Human brain tumour cell strains with deficient host-cell reactivation of N-methyl-N'-nitro-N-nitrosoguanidine-damaged adenovirus 5, Nature, 279:797.

Day, R.S., III, Ziolkowski, C.H.J., Scudiero, D.A., Meyer, S. A., Mattern, M.R., 1980a, Human tumor cell strains defective in the repair of alkylation damage, Carcinogenesis, 1:21.

Day, R.S., III, Yarosh, D.B., and Ziolkowski, C.H.J., 1984, Relationship of methyl purines produced by MNNG in adenovirus 5 DNA to viral inactivation in repair-deficient (Mer-) human tumor cell strains. Mutation Res., 131: 45.

Day, R.S., III, Ziolkowski, C.H.J., Scudiero, D.A., Meyer, S. A., Lubiniecki, A.S., Girardi, A.J., Galloway, S.M., and Bynum, G.D., 1980b, Defective repair of alkylated DNA by human tumour and SV40-transformed human cell strains, Nature, 288:724.

Day, R.S., III, Babich, M.A., Yarosh, D.B., and Scudiero, D. A., 1987, The role of O^6-methylguanine in human cell killing, sister-chromatid exchange induction and mutagenesis: a review. J. Cell. Sci. Suppl., 6:333.

Deinhardt, F., 1980, Biology of primate retroviruses, in: "Viral Oncology," G. Klein, ed., Raven Press, New York, p. 357.

Diaz, M.O., Le Beau, M.M., Pitha, P., and Rowley, J.D., 1986, Interferon and c-ets-1 genes in the translocation

(9;11)(p22;q23) in human acute monocytic leukemia, Science, 231:265.

Diaz, M.O., Zieman, S., Le Beau, M.M., Pitha, P., Smith, S. D., Chilcote, R., and Rowley, J.D., 1988, Homozygous deletion of the alpha- and beta-1-interferon genes in human leukemia and derived cell lines. Proc. Natl. Acad. Sci. USA, 85:5259.

Domoradzki, J., Pegg, A.E., Dolan, M.E., Maher, V.M., and McCormick, J.J., 1984, Correlation between O^6-methylguanine-DNA-methyltransferase activity and resistance of human cells to the cytotoxic and mutagenic effect of N-methyl-N'-nitro-N-nitrosoguanidine, Carcinogenesis, 5: 1641.

Erickson, L.C., Laurent, G., Sharkey, N.A., and Kohn, K.W., 1980a, DNA cross-linking and monoadduct removal in nitrosourea-treated human tumour cells. Nature, 288:727.

Erickson, L.C., Bradley, M.O., Ducore, J.M., Ewig, R.A.G., and Kohn, K.W., 1980b, DNA crosslinking and cytotoxicity in normal and transformed human cells treated with antitumor nitrosoureas. Proc. Natl. Acad. Sci. USA, 77:467.

Fox, M., Brennand, J., and Margison, G.P., 1987, Protection of Chinese hamster cells against the cytotoxic and mutagenic effects of alkylating agents by transfection of the Escherichia coli alkyltransferase gene and a truncated derivative. Mutagenesis, 2:491.

Freeman, A.E., and Hoffman, R.M., 1986, In vivo-like growth of human tumors in vitro. Proc. Natl. Acad. Sci. USA, 83: 2694.

Giard, D.J., Aaronson, S.A., Todaro, G.J., Arnstein, R., Kersey, J.H., Dosik, H., and Parks, W.P., 1973, In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors, J. Natl. Cancer Inst., 51:1417.

Gibson, N.W., Hartley, J.A., Strong, J.M., and Kohn, K.W., 1986, 2-chloroethyl(methylsulfonyl)methanesulfonate(NSC-338947) a more selective DNA alkylating agent than the chloroethylnitrosoureas. Cancer Res., 46:553.

Haley, J., Whittle, N., Bennett, P., Kinchington, D., Ullrich, A., and Waterfield, M., 1987, The human EGF receptor gene: structure of the 110 kb locus and identification of sequences regulating its transcription. Oncogene Res., 1:375.

Ishizaki, K., Tsujimura, T., Fujio, C., Yangpei, Z., Yawata, H., Nakabeppu, Y., Sekiguchi, M., and Ikenaga, M., 1986, Expression of the truncated E. coli O^6-methylguanine methyltransferase gene in repair-deficient human cells and restoration of cellular resistance to alkylating agents. Mutation Res., 184:121.

Jakobovits, E.B., Majors, J.E., and Varmus, H.E., 1984, Hormonal regulation of the Rous sarcoma virus src gene via a heterologous promoter defines a threshold dose for cellular transcription. Cell, 38:757.

James, C.D., Carlbom, E., Dumanski, J.P., Hansen, M., Nordenskjold, M., Collins, V.P. and Cavenee, W.K., 1988, Clonal genomic alterations in glioma malignancy stages. Cancer Res., 48:5546.

Jones, N., and Shenk, T., 1979, Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell, 17:683.

Kataoka, H., Hall, J., and Karran, P., 1986, Complementation of sensitivity to alkylating agents in Escherichia coli

and Chinese hamster cells by expression of a cloned bacterial repair gene. EMBO J., 5:3195.
Kinzler, K.W., Bigner, S.H., Bigner, D.D., Trent, J.M., Law, M.L., O'Brien, S.J., Wong, A.J., and Vogelstein, B., 1987, Identification of an amplified, highly expressed gene in a human glioma. Science, 236:70.
Libermann, T.A., Nusbaum, H.R., Razon, N., Kris, R., Lax, I., Soreq, H., Whittle, N., Waterfield, M.D., Ullrich, A., and Schlessinger, J., 1985, Amplification, enchanced expression, and possible rearrangement of EGF receptor gene in primary human tumors of glial origin. Nature, 313:144.
Mark, D.F., Lu, S.D., Creasey, A.A., Yamamoto, R., and Lin, L.S., 1984, Site-specific mutagenesis of the human fibroblast interferon gene. Proc. Natl. Acad. Sci. USA, 81:5662.
Mosmann, T.J., 1983, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immun. Methods, 65:55.
Nister, M., Libermann, T.A., Betsholtz, C., Petterson, M., Clesson-Welsh, L., Heldin, C.H., Schlessinger, J., and Westermark, B., 1988, Expression of messenger RNAs for platelet-derived growth factor and transforming growth factor-alpha and their receptors in human malignant cell lines. Cancer Res., 48:3910.
Ponten, J., 1975, Neoplastic human glia cells in culture. in: "Human Tumor Cells in Vitro", J. Fogh, ed., Plenum Press, New York. p 175.
Resnitzky, D., Yarden, A., Zipori, D., and Kimchi, A., 1986, Autocrine beta-related interferon controls c-myc suppression and growth arrest during hematopoietic cell differentiation. Cell, 46:31.
Samson, L., Derfler, B., and Waldstein, E.A., 1986, Suppression of human DNA alkylation-repair defects by Escherichia coli DNA-repair genes. Proc. Natl. Acad. Sci. USA, 83: 5607.
Sariban, E., Kohn, K.W., Zlotogorski, C., Laurent, G., D'Incalci, M., Day, R.S., III, Smith, B.H., Kornblith, P.L., and Grickson, L.C., 1987, DNA cross-linking responses of human malignant glioma cell strains to chloroethylnitrosoureas, cisplatin, and diaziquone. Cancer Res., 47:3988.
Scudiero, D.A., Meyer, S.A., Clatterbuck, B.E., Mattern, M. R., Ziolkowski, C.H.J., and Day, R.S., III, 1984a, Relationship of DNA repair phenotypes of human fibroblast and tumor strains to killing by N-methyl-N'-nitro-N-nitrosoguanidine, Cancer Research, 44:961.
Scudiero, D.A., Meyer, S.A., Clatterbuck, B.E., Mattern, M. R., Ziolkowski, C.H.J., and Day, R.S., III, 1984b, Sensitivity of human cell strains having different abilities to repair O^6-methylguanine in DNA to inactivation by alkylating agents including chloroethylnitrosoureas. Cancer Res., 44:2467.
Shows, T.B., Sakaguchi, A.Y., Naylor, S.L., Goeddel, D.V., and Lawn, R.M., 1982, Clustering of leucocyte and interferon genes on human chromosome 9. Science, 218:373.
Sklar, R., and Strauss, B., 1981, Removal of O^6-methylguanine from DNA of normal and xeroderma pigmentosum-derived lymphoblastoid cell lines, Nature, 289:417.
Watatani, M., Ikenaga, M., Hatanaka, T., Kinuta, M., Takai, S., Mori, T. and Kondo, S., 1985, Analysis of N-methyl

N'-nitro-N-nitrosoguanidine (MNNG)-induced DNA damage in tumor cells strains from Japanese patients and demonstration of MNNG hypersensitivity of Mer- xenografts in athymic nude mice. Carcinogenesis, 6:549.

Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C.H., Huang, J.S., and Deuel, T.F., 1983, Platelet-derived growth factor is structurally related to the putative transforming protein p28 sis of simian sarcoma virus. Nature, 304:35.

Wilson, C.B., 1976, Chemotherapy of brain tumors, in: "Advances in Neurology, Vol. 15, Neoplasia in the Central Nervous System," R.A. Thompson and J.R. Green, eds., Raven Press, New York. p. 361.

Wong, A.J., Bigner, S.H., Bigner, D.D., Kinzler, K.W., Hamilton, S.R., and Vogelstein, B., 1987, Increased expression of the epidermal growth factor receptor gene in malignant gliomas is invariably associated with gene amplification. Proc. Natl. Acad. Sci. U.S.A., 84:6899.

Yamazaki, H., Fukui, Y., Ueyama, Y., Tamaoki, N., Kawamoto, T., Taniguchi, S., and Shibuya, M., 1988, Amplification of the structurally and functionally altered epidermal growth factor receptor gene (c-erbB) in human brain tumors. Mol. Cell. Biol., 8:1816.

Yarosh, D.B., Rice, M., Day, R.S., III, Foote, R.S., and Mitra, S., 1984a, O^6-methylguanine-DNA methyltransferase in human cells. Mutat. Res., 131:27.

Yarosh, D.B., Scudiero, D.A., Ziolkowski, C.H.J., Rhim, J.S., and Day, R.S., III. , 1984b, Hybrids between human tumor cells differing in repair of MNNG-produced DNA damage. Carcinogenesis, 5:627.

Yarosh, D.B., Scudiero, D.A., Yagi, T., and Day, R.S., III, 1985, Human tumor cell strains both unable to repair O^6-methylguanine and hypersensitive to killing by human alpha and beta interferons. Carcinogenesis, 6:883.